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OPTIMIZATION OF PHYSICOCHEMICAL FACTORS FOR MICROALGAL BIOMASS USING INDIGENOUSLY ISOLATED MICROALGAE

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ABSTRACT

In the current global energy crisis scenario, microalgae are considered an efficient feedstock for the green synthesis of biofuels. Therefore, it is a dire need of time to screen, select and optimizes the growth conditions of indigenous microalgal strains for enhanced biofuel production. This study was designed to optimize the physicochemical conditions required for the growth of indigenous microalgal strains (n=10). All strains were grown in BG-11 media and optimized for different physical requirements, i.e., pH, temperatures, light intensity, NaCl, and chemical requirements, i.e., carbon source (glucose, glycerol, maltose, acetic acid), nitrogen source (urea, ammonium chloride, potassium nitrate, sodium nitrate), Nitrogen levels, phosphorus level, sodium bicarbonate concentration, and CO_2. All strains were grown for 10 days, and growth was monitored by measuring optical density at 650 nm. Results revealed that out of 10 strains, the optimum growth of most of the strains was at 35ºC (5/10, 50%), pH 7 (8/10, 80%), light intensity of 2000 lux (5/10, 50%), and 1% NaCl (5/10, 50%). Similarly, most of the strains had higher growth in media supplemented with 40 mM of $NaNO_3$ (7/10, 70%), 2 mM phosphorus (5/10, 50%), 1% glycerol (9/10, 90%) in autotrophic conditions, and 1% glucose (5/10, 50%) in mixotrophic conditions. The overall finding of the study revealed that S*cenedesmus* AIN01 showed significantly higher growth at 35ºC, pH 7, 2000 lux light, 1% NaCl, 40 mM $NaNO_3$, 1.5 mM phosphorus, 1% glycerol, and 0.1% bicarbonate as compared to other strains grown at their optimum conditions. It is concluded that the strains in this study, especially S*cenedesmus* AIN01, may be grown using their optimum physicochemical conditions for enhanced growth for subsequent use in biofuel production.

Keywords: Light intensity, Microalgae, Nitrogen sources, Salinity, Temperature.

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INTRODUCTION

Microalgae are microscopic, photosynthetic organisms, capable of fixing carbon dioxide from the air and absorbing water from the soil to synthesize energy and biomass using solar energy (Dolganyuk *et al.,* 2020). The important prokaryotic microalgae are Cyanobacteria (Cyanophyceae) whereas eukaryotic microalgae includes green algae (Chlorophyta) and diatoms (Li *et al.,* 2008). Biomass produced by microalgae is a valuable source for extracting biologically active compounds such as lipids for biofuel production. Fossil fuels have a limited supply, and burning fossil fuels plays a major role in global warming. Photosynthetic microalgae are promising organisms as feedstock to produce biodiesel. They can achieve higher growth rates and convert sun energy into effective biomolecules such as lipids which can be converted into biodiesel (Ghosh *et al.,* 2016).

Biofuel production from microalgal strains is preferred because they have higher growth rates and ultimately can accumulate more lipids than oil crops. They can better adapt to local environmental conditions. To date 40,000 species of microalgae have been identified. Selecting suitable microalgae and an appropriate cultivation scheme is the key to economic feasibility of biofuel from microalgae (Gilmour, 2019). Current studies revealed that the growth rate of microalgae could be enhanced by proper cultivation conditions such as temperature, pH, light intensity, salinity, nitrogen sources, nitrogen levels, phosphorus levels, autotrophy, heterotrophy and CO_2 concentration. The growth of microalgae cells increases with the increase in temperature. The optimum temperature for most microalgae was 30°C (Chinnasamy *et al.,* 2009). A

decrease in temperature from 30°C to 25°C, reduced the growth and lipid level from 14.7 to 5.9% (Converti *et al.,* 2009). The pH affects the algal metabolism by varying the availability of nutrients in the medium. It also affects carbon dioxide availability and solubility. Algal cells take up more carbon from inorganic sources as the level of pH increases in culture conditions. However neutral pH is considered optimal for algal growth (Zhang *et al.*, 2019). Salinity is an environmental factor that promotes the growth of algae and limits the invading microbes (Bartley *et al.,* 2013). Algal cells prefer neutral salinity. Changes in salinity affect nutritional composition and growth rate as well. If salinity increases, algae lipid contents also increase, and growth is reduced. (Hu *et al.,* 2008).

Nitrogen is being utilized to produce of nucleic acid and protein components of cells. The nitrogen deficiency reduces carbon dioxide fixation, lesser chlorophyll contents and less oxygen evolution by algal cells (Pulgarin *et al.*, 2021). Phosphorus is involved in energy carrier molecules such as ATP, part of the nucleic acid backbone and is involved in cell membrane integrity as phospholipids. So it is important for cells normal growth and development**.** Phosphorus limitation leads to increased biosynthesis of lipids and a decreased in biomass and chlorophyll level in microalgae (Rowbotham *et al.*, 2012). Carbon is an important and major nutrient required for growth. It plays an important role in photosynthesis. Carbon can be provided to autotrophic growth modes such as bicarbonate, carbonate, and CO_2. At the same time, carbon sources for heterotrophic algal growth are glucose, glycerol, maltose and acetate. The concentration of carbon affects lipid distribution within the cell. However, elevated CO_2 level decreases protein contents and increases carbohydrate contents (Gao *et al.,* 2021).

Various strategies can be employed for the large-scale production of phototrophic microalgae. Large scale, indoor algae biomass production is possible using highly controlled photo-bioreactors or fermenters. Lipid accumulation by microalgae can be affected by various factors including light intensity, temperature, pH, salinity, nitrogen source concentration and mineral salts (Yeesang and Cheirsilp, 2011). The present study evaluated different physicochemical factors on isolated microalgae to optimize growth rates for better biofuel production. BG-11 media in 96 well plates were utilized as the base for microalgae growth; three physical factors (Temperature, pH and light intensity) were investigated for biomass production. Seven chemical factors (nitrogen sources, nitrogen concentration, phosphorus concentration, carbon sources under autotrophic and heterotrophic conditions, salinity, bicarbonate concentration and CO_2 concentration) were investigated. Results may help achieve higher microalgae growth rates for better biofuel production.

MATERIALS AND METHODS

Characterized microalgae isolates: Indigenous micro-algal strains were isolated from soil and water samples of three districts (Lahore, Sheikhupura and Faisalabad) and meteorological conditions of these areas vary during the year as temperature fluctuates from 5℃ to 40℃, average humidity 36%, and the average annual precipitation level around 628.8 millimeters. Mostly soil in three districts is clay loam and having pH 8 with electrical conductivity (EC) e values around 3.5 d Sm^{-1} (Ahmad and Rasul, 2008). The total number of 180 isolates (n=180) were obtained from 60 samples (n=30 soil and n=30 water). In order to isolate diverse microalgae, soil samples were randomly collected from the fields along with canal banks and water courses, while water samples (with a green tinge) were collected from ponds, left-over water after the closure of water courses and canals in a three mentioned districts. Sample sites and GPS coordinates of isolated microalgae are presented in table 1. Out of 180 isolates, only 10 isolates were selected on the basis of growth, shape and presence of lipids. These 10 isolates were further analyzed by various biochemical tests (Anthrone assay, Bradford assay and microvanilline assay) for quantification of carbohydrate, protein and lipid contents. After biochemical characterization, these isolates were subjected to growth optimization by physicochemical factors.

Table 1: Water and soil samples collection sites and GPS coordinates of isolated microalgae

Sr. No.	Isolate	Location	GPS Coordinates
1	**AIN01**	Kot Mahmood, Sheikhupura	31.453044°N, 74.059173°E
2	**AIN09**	Chak 559 GB, Sheikhupura	31.45098°N, 73.64606°E
3	**AIN24**	Hiran Minar Park, Sheikhupura	31.74206°N, 73.95463°E
4	**AIN48**	Shadab Village, Sheikhupura	31.53335°N, 74.10671°E
5	**AIN63**	Khan Pur, Sheikhupura	31.675881°N, 74.099226°E
6	**AIN68**	Chak 78 GB (west), Faisalabad	31.293318°N, 73.139397°E
7	**AIN79**	Chak 236RB Kajlay, Faisalabad	31.314546°N, 73.109230°E
8	**AIN85**	Gatwala Park Lake, Faisalabad	31.480783°N, 73.211357°E
9	**AIN93**	Jallo Park Lake, Lahore	31.571749°N, 74.468455°E
10	**AIN95**	Jallo Park, Lahore	31.573181°N, 74.464953°E

Physical requirements: Temperature, light and pH were optimized by cultivating microalgae at different temperatures levels (20°C, 25°C, 30°C and 35°C), four different light regimes (500, 1000, 1500 and 3000 lux) and pH ranges (6,7,8,9) respectively. BG-11 sterilized medium was transferred to 96 well micro-titration plates (200µL in each well) and each well was inoculated with standard volume (20µL) of respective microalgae at same OD value. For temperature optimization, plates were incubated at respective temperatures (20°C, 25°C, 30°C and 35°C) at 1000 lux light for a period of 15 days by providing light period (16h light, 8h dark). Plates were incubated at 30±2°C respective light levels (500, 1000, 1500 and 3000 lux) for a period of 15 days by providing light period (16h light, 8h dark) for level of light. For pH effect, plates were incubated at 30±2°C at 1000 lux for a period of 15 days by providing light period (16h light, 8h dark). For all physical factors, OD values at 680 nm were recorded daily by using ELISA reader (Politaeva *et al.,* 2017).

Salinity: To check the effect of salinity, BG-11 medium supplemented with following concentrations of sodium chloride (0.5%, 1%, 1.5% and 2%) were evaluated following the method of Luangpipat and Chisti with minor modifications and plates were incubated at 30±2°C at 1000 lux for a period of 15 days by providing light period (16h light, 8h dark). For results evaluation, OD values at 680 nm were recorded daily by using ELISA reader (Luangpipat and Chisti, 2017).

Chemical requirements: Chemical requirements were optimized by testing different concentrations of nutrients. In order to optimize chemical requirement protocol was followed according to Colla *et al.,* (2007). Different sources of nitrogen [Sodium Nitrate (20 mM), Potassium Nitrate (20 mM), Ammonium Chloride (20 mM) and Urea (20 mM)] and concentration of sodium nitrate (10, 20, 30 and 40 mM) were prepared in BG-11 medium using respective salts, prepared medium with different sources and levels of nitrogen were autoclaved in independent medium flasks. In order to optimize best phosphorus concentration for micro algal growth, different concentrations of di-potassium hydrogen phosphate (0.5, 1, 1.5 and 2 mM) were tested. In order to optimize carbon requirements for algal growth and to evaluate mixotrophic and heterotrophic microalgae growth potential, glucose, glycerol, fructose and acetic acid (1% in BG- 11 medium) in the presence and absence of light were used. To check the effect of dissolved CO_2 different concentrations of sodium bicarbonate (0.1, 0.2, 0.3 and 0.4 %) in BG-11 medium were evaluated. To check the effect of external carbon source, 4% CO_2 was used. Micro titration plates containing BG-11 medium were prepare and placed in a CO_2 incubator. Plates were incubated at 30±2°C temperature and 1000 lux light for a period of 15 days by providing light period (16h light, 8h dark). OD values at 680 nm were recorded daily by using ELISA reader.

Statistical analysis: Experiments were conducted by one factor one time analysis. The data obtained from experimental results were analyzed statistically by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT) post hoc analysis using the software statistical package for the social sciences (SPSS).

RESULTS

Optimization of growth: Different physicochemical parameters were evaluated for their effect on growth of different microalgae. Growth of microalgae at different temperatures is shown in table 2 and fig. 1A. Algal isolates showed optimum growth at 25ºC were (AIN63, AIN93, AIN95), 30ºC (AIN68, AIN 85), 35ºC (AIN01, AIN09, AIN 24, AIN48 and AIN79). Algal isolates (AIN09, AIN48, AIN63, AIN68, AIN79, AIN85 and AIN93) differ significantly with others while algal isolates (AIN01, AIN24 and AIN95) differ non-significantly with others. Growth of microalgae at different pH is shown in table 2 and fig. 1B. Algal isolates showed optimum growth at pH 6 (AIN93, AIN95), pH 7 (AIN01, AIN09, AIN24, AIN48, AIN 63, AIN68, AIN79, AIN85), while none of isolates showed optimum growth at pH 8 and 9. Algal Isolates (AIN01, AIN09, AIN48, AIN63, AIN68, AIN79, AIN85, AIN93 and AIN95) differs significantly with others while algal isolates (AIN24) differs non-significantly with others. Growth of microalgae at different levels of light was evaluated and algal isolates showed optimum growth at 1000Lux (AIN85, AIN93), at 1500 lux (AIN68, AIN79, AIN95) and at 2000 lux (AIN01, AIN09, AIN24, AIN48 and AIN63). Algal isolates (AIN01, AIN09, AIN24, AIN48, AIN63, AIN68, AIN79, AIN85, AIN93 and AIN95) differ significantly with others (Table 2 and Fig. 1C).

Growth of microalgae at different sources of nitrogen is shown in table 4 and fig. 2A. Algal isolates showed optimum growth at urea (20mM) (AIN63, AIN68, AIN79), none of isolate showed optimum growth with the supplementation of NH_4Cl (20mM), while Algal isolates showed optimum growth at KNO_3 (20mM) (AIN01, AIN09, AIN24, AIN93). Algal isolates showed optimum growth at $NaNO_3$ (AIN48, AIN85, AIN95). Growth of microalgae at different concentration of nitrogen was evaluated. Algal isolates showed optimum growth at N1 (40mM) (AIN01, AIN09, AIN48, AIN63, AIN79, AIN85, AIN95), at N2 (30mM) (AIN93) and at N4 (10mM) (AIN24, AIN68). Algal isolates (AIN01, AIN09, AIN48, AIN63, AIN85 and AIN95) while algal isolates that differs non-significantly (AIN24, AIN68, AIN79 and AIN93) with others (Table 4 and Fig. 2B).

Table 2: Effect of Physical factors on growth of selected algal isolates (n=10) as determined by OD (Mean ± S.D) at 650 nm after 10 days of incubation

Sr. No.	Isolate	Effect of pH		Effect of Temp (°C)		Light Level (lux)	
1	**AIN01**	6	1.38±.08[ab]	20	1.26±.04[a]	500	0.70±.05[a]
		7	1.79±.02 [c]	25	1.23±.04[a]	1000	1.53±.04[b]
		8	1.29±.08 [a]	30	1.26±.02[a]	1500	1.53±.01[b]
		9	1.43±.08 [b]	35	1.34±.01[a]	2000	1.64±.04[b]
2	**AIN09**	6	0.88±.10 [a]	20	1.09±.05[a]	500	0.90±.04[a]
		7	1.47±.19 [b]	25	1.12±.05[a]	1000	1.14±.05[b]
		8	0.90±.04 [a]	30	1.09±.08[a]	1500	1.20±.04[b]
		9	0.83±.28 [a]	35	1.22±.02[b]	2000	1.28±.07[b]
3	**AIN024**	6	0.45±.13 [a]	20	0.31±.02[a]	500	.39±.06[ab]
		7	0.49±.05 [a]	25	0.31±.04[a]	1000	.47±.05[bc]
		8	0.48±.08 [a]	30	0.31±0.05[a]	1500	0.31±.04[a]
		9	0.48±.07 [a]	35	0.34±.01[a]	2000	0.55±.02[c]
4	**AIN048**	6	0.74±.14 [a]	20	0.77±.02[a]	500	0.53±.05[a]
		7	0.95±.09 [b]	25	0.90±.04[ab]	1000	0.90±.05[b]
		8	0.66±.12 [a]	30	0.78±.03[b]	1500	0.99±.04[b]
		9	0.59±.11[a]	35	1.07±.05[b]	2000	1.09±.01[b]
5	**AIN63**	6	0.57±.33[a]	20	0.71±.03[a]	500	0.26±.07[a]
		7	1.30±.07[b]	25	0.73±.05[b]	1000	0.57±.04[b]
		8	0.71±.23[a]	30	0.72±.11[c]	1500	0.56±.08[b]
		9	0.86±.21[a]	35	0.07±.06[c]	2000	0.65±.04[b]
6	**AIN68**	6	0.70±.21[a]	20	0.76±.02[a]	500	0.27±.09[a]
		7	1.33±.02[b]	25	0.72±.08[b]	1000	.93±.04[bc]
		8	0.79±.15[a]	30	0.76±.03[b]	1500	0.95±.08[c]
		9	0.78±.11[a]	35	0.53±.09[b]	2000	0.74±.01[b]
7	**AIN79**	6	0.81±.17[a]	20	1.00±.01[a]	500	0.33±.05[a]
		7	1.20±.05 [b]	25	0.97±.07[a]	1000	0.98±.08[b]
		8	0.80±.05 [a]	30	1.00±0.08[a]	1500	1.02±.04[b]
		9	0.67±.04 [a]	35	1.19±.04[b]	2000	1.01±.04[b]
8	**AIN85**	6	1.15±.08 [ab]	20	0.92±.02 [a]	500	0.60±.04[a]
		7	1.38±.14[b]	25	0.72±.03[ab]	1000	1.15±.08[b]
		8	1.07±.24[a]	30	0.93±.17[ab]	1500	1.07±.04[ab]
		9	0.97±.23[a]	35	0.88±.05[b]	2000	1.02±.04[ab]
9	**AIN93**	6	1.36±.04 [c]	20	0.918±.02[a]	500	0.38±.05[a]
		7	.92± .01[ab]	25	1.05±.01[b]	1000	1.08±.06[b]
		8	1.02±.05 [b]	30	0.92±.20[bc]	1500	1.02±.05[b]
		9	0.88±.09 [a]	35	0.515±.01[c]	2000	1.04±.04[b]
10	**AIN95**	6	1.11±.03[d]	20	0.806±.01[a]	500	0.48±.06[a]
		7	0.58±.01[a]	25	0.893±.01[a]	1000	0.94±.05[b]
		8	0.78±.03[c]	30	0.81±0.16[a]	1500	0.97±.04[b]
		9	0.73±.03[a]	35	0.771±.05[a]	2000	0.95±.09[b]

[a,b,c,d] Different superscript shows statistically significant difference ($p < 0.05$) in different rows of same column.

Growth of microalgae at different concentrations of sodium chloride is shown in table 3 and fig. 1D. Algal isolates showed optimum growth at 0.5% NaCl (AIN68, AIN79 and AIN85), 01% NaCl (AIN09, AIN48, AIN63 and AIN95), 2% NaCl (AIN24 and AIN93) and 3% NaCl (AIN01). Algal isolates (AIN01, AIN09, AIN48, AIN63, AIN68, AIN79, AIN85 and AIN95) differ significantly with others while algal isolates that differs non-significantly (AIN24, AIN93) with others.

Growth of microalgae at different concentration of phosphorus is shown in table 4 and fig. 2C. Algal isolates showed optimum growth at P1 (AIN01, AIN09, AIN48, AIN63, AIN68, AIN85) and at P2 (AIN24, AIN79, AIN93) respectively. Algal isolates (AIN01, AIN09, AIN24, AIN48, AIN63, AIN68, AIN79, AIN93, AIN95) differ significantly with others while algal isolates that differs non-significantly (AIN85) with others. Growth of microalgae at different sources of carbon in absence of light is shown in table 4 and fig. 2D. Algal isolates showed optimum growth at glycerol (AIN01, AIN09, AIN24, AIN48, AIN63, AIN68, AIN79, AIN85, AIN93 and AIN95), while none of isolate showed optimum growth at maltose and acetic acid. Algal isolates (AIN01, AIN09, AIN24, AIN48, AIN63, AIN68, AIN79, AIN85, AIN93) significantly with others while algal isolates that differs non-significantly (AIN95) with others. Growth of microalgae at different sources of carbon in presence of light is shown in table 4 and fig. 2E. Algal isolates showed optimum growth at glucose (AIN48, AIN63, AIN68, AIN79 and AIN95), glycerol (AIN24) and acetic acid (AIN01, AIN09, AIN85 and

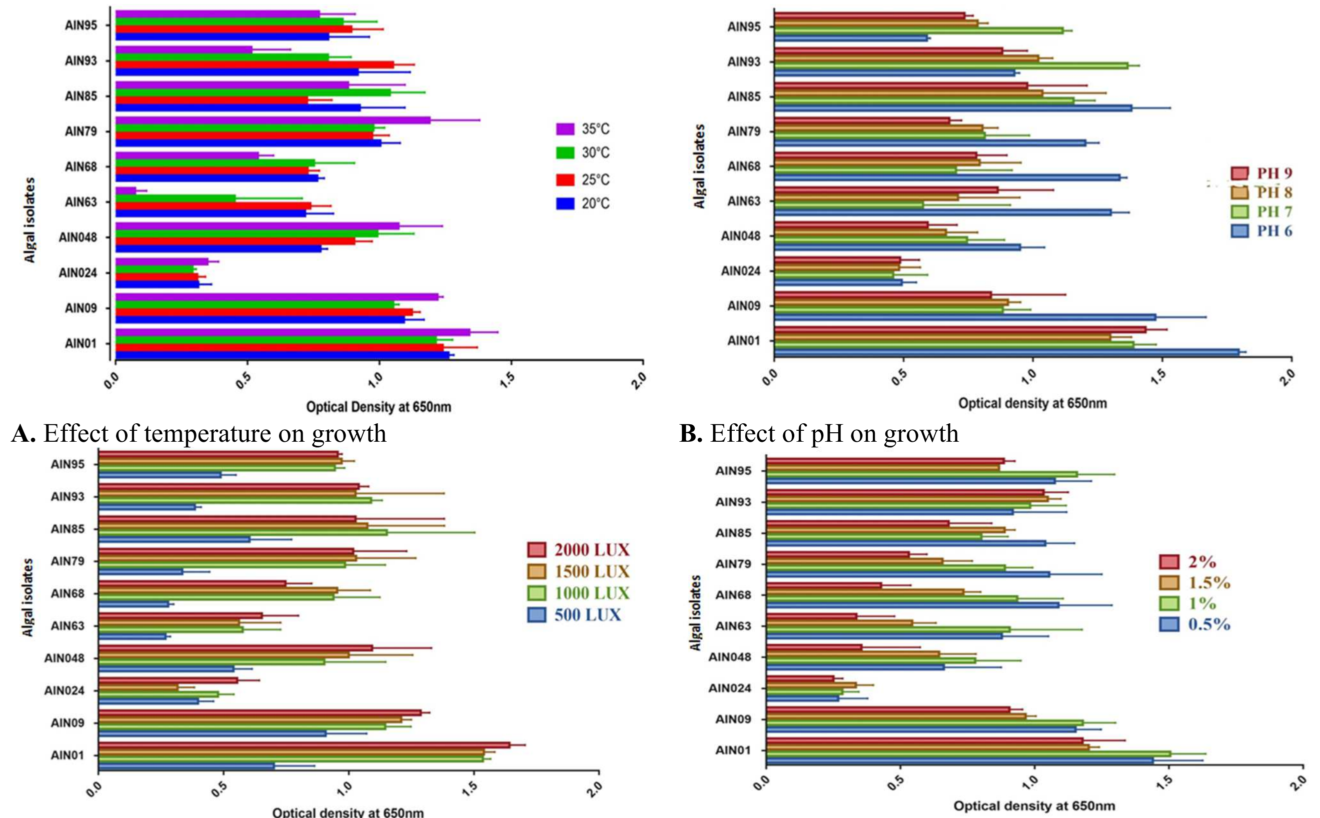
AIN93). Algal isolates (AIN01, AIN09, AIN48, AIN63, AIN68, AIN79, AIN93 and AIN95) significantly with others while algal isolates that differs non-significantly (AIN24, AIN85) with others.

Growth of microalgae at different concentrations of sodium bicarbonate is shown in table 4 and fig. 2F. Algal isolates showed optimum growth at 0.1% sodium bicarbonate (AIN01, AIN09, AIN24, AIN48, AIN63, AIN68, AIN79, AIN85 and AIN95) and 0.2% sodium bicarbonate (AIN93). While none of isolate showed optimum growth at 0.3% and 0.4% sodium bicarbonate. Algal isolates (AIN01, AIN09, AIN48, AIN63, AIN68, AIN79 and AIN95) significantly with others while algal isolates that differs non-significantly (AIN24, AIN85, AIN93) with others. Growth of microalgae at 4% CO_2 is shown in table 4 and fig. 2G. All algal isolates showed optimum growth ($p < 0.05$) at 4% CO_2 then growth without CO_2.

Table 3: Effect of sodium chloride on growth of selected algal isolates (n=10) as determined by OD (Mean ± S.D.) at 650 nm after 10 days.

Sr. No.	Isolate	Growth (Mean ± S.D) at NaCl Conc.			
		0.5%	1.0%	2.0%	3.0%
1	**AIN01**	1.44± 0.18 ^{b}	1.50± 0.13 ^{b}	1.20± 0.03 ^{a}	1.18± 0.15 ^{a}
2	**AIN09**	1.15± 0.09 ^{b}	1.18± 0.12 ^{b}	0.96± 0.03 ^{a}	0.91± 0.04 ^{a}
3	**AIN24**	0.27± 0.11 ^{a}	0.28± 0.06 ^{a}	0.33± 0.06 ^{a}	0.25± 0.03 ^{a}
4	**AIN48**	0.66± 0.21 ^{b}	0.77± 0.16 ^{b}	0.64± 0.13 ^{a}	0.35± 0.21 ^{a}
5	**AIN63**	0.88± 0.17 ^{b}	0.90± 0.26 ^{b}	0.54± 0.08 ^{a}	0.34± 0.14 ^{a}
6	**AIN68**	1.08± 0.19 ^{c}	0.93± 0.17^{bc}	0.73± 0.06 ^{b}	0.43± 0.11 ^{a}
7	**AIN79**	1.05± 0.19 ^{b}	0.89± 0.10 ^{b}	0.66± 0.11 ^{a}	0.53± 0.06 ^{a}
8	**AIN85**	1.03± 0.10 ^{c}	0.80± 0.09 $^{a b}$	0.88± 0.03 $^{b c}$	0.67± 0.16 ^{c}
9	**AIN93**	0.91± 0.20 ^{a}	0.98± 0.13 ^{a}	1.04± 0.04 ^{a}	1.03± 0.09 ^{a}
10	**AIN95**	1.07± 0.13 ^{b}	1.15± 0.14 ^{b}	0.86± 0.00 ^{a}	0.89± 0.042 ^{a}

^{a,b,c,d} Different superscript shows statistically significant difference ($p < 0.05$) in different columns of same row.



A. Effect of temperature on growth

B. Effect of pH on growth

C. Effect of light intensity on growth

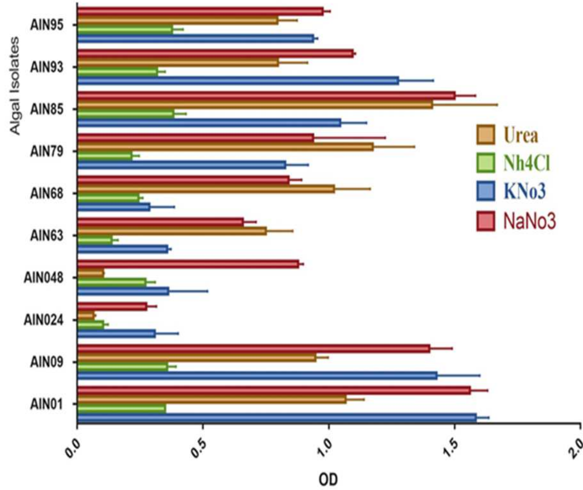
D. Effect of NaCl concentrations

Fig. 1. Effect of physical factors and different concentrations of NaCl on algal isolates at day 10 as determined by optical density (OD=650)

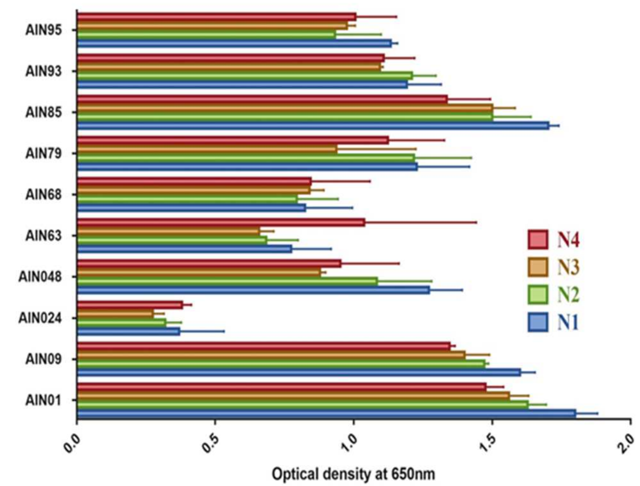
Table 4: Effect of Chemical factors on growth of selected algal isolates (n=10) as determined by optical density (Mean ± S.D) at 650 nm after 10 days

Sr. No.	Isolate	Nitrogen sources (20mM)		Nitrogen Concentration (mM)		Effect of Phosphorus (mM)		Carbon Source			Effect of Sodium bicarbonate		Effect of CO_2	
								Conc. 1%	Absence of light	Presence of light				
1	**AIN01**	Urea	1.06±.07^{b}	N1(40)	1.79±.04^{c}	P1(2.0)	1.56±.05^{c}	Glucose	0.15±.02^{a}	0.89±.23^{a}	0.1%	1.37±.10^{c}		
		NH_4Cl	.34± .00^{a}	N2(30)	1.62±.04^{ab}	P2(1.5)	1.61±.01^{c}	Glycerol	0.47±.13^{c}	1.19±.09^{bc}	0.2%	1.27±.08^{bc}	5%	1.33±.05
		KNO_3	1.58±.05^{c}	N3(20)	1.56±.04^{ab}	P3(1.0)	1.29±.02^{a}	Maltose	0.34±.02^{b}	1.05±.24^{ab}	0.3%	1.18±.08^{ab}		
		$NaNO_3$	1.56±.04^{c}	N4(10)	1.47±.02^{a}	P4(0.5)	1.42±.07^{b}	Acetic Acid	0.23±.01^{a}	1.41±.06^{c}	0.4%	1.12±.05^{a}	Normal	1.23±.07
2	**AIN09**	Urea	.94± .05^{b}	N1(40)	1.60±.05^{c}	P1(2.0)	1.40±.09^{c}	Glucose	0.24±.04^{a}	0.96±.23^{ab}	0.1%	1.13±.00^{b}		
		NH_4Cl	0.35±.04^{a}	N2(30)	1.47±.05^{b}	P2(1.5)	1.26±.14^{bc}	Glycerol	0.49±.14^{b}	1.04±.07^{ab}	0.2%	0.97±.12^{a}	5%	1.39±.04
		KNO_3	1.43±.17^{c}	N3(20)	1.39±.04^{ab}	P3(1.0)	1.14±.09^{ab}	Maltose	0.31±.03^{a}	0.84±.13^{a}	0.3%	0.97±.01^{a}		
		$NaNO_3$	1.39±.04^{c}	N4(10)	1.35±.04^{a}	P4(0.5)	1.041±.04^{a}	Acetic Acid	0.31±.04^{a}	1.06±.03^{b}	0.4%	0.98±.07^{a}	Normal	1.29±.05
3	**AIN024**	Urea	0.06±.01^{a}	N1(40)	0.37±.06^{a}	P1(2.0)	0.30±.06^{a}	Glucose	0.09±.02^{a}	0.26±.08^{a}	0.1%	0.35±.05^{a}		
		NH_4Cl	0.10±.01^{a}	N2(30)	0.32±.06^{a}	P2(1.5)	0.46±.01^{b}	Glycerol	0.32±.11^{c}	0.36± 0.1^{a}	0.2%	0.27±.04^{a}	5%	0.44±.05
		KNO_3	0.30±.09^{b}	N3(20)	0.27±.02^{a}	P3(1.0)	0.39±.06^{ab}	Maltose	0.21±.00^{b}	0.31±.05^{a}	0.3%	0.28±.03^{a}		
		$NaNO_3$	0.27±.02^{b}	N4(10)	0.37±.05^{a}	P4(0.5)	0.44±.05^{b}	Acetic Acid	0.22±.04^{b}	0.29±.02^{a}	0.4%	0.29±.00^{a}	Normal	0.34±.06
4	**AIN048**	Urea	0.10±.00^{a}	N1(40)	1.27±.08^{b}	P1(2.0)	0.88±.24^{b}	Glucose	0.19±.01^{a}	1.11±.09^{c}	0.1%	0.62±.04^{ab}		
		NH_4Cl	.27±.03^{b}	N2(30)	1.08±.05^{ab}	P2(1.5)	0.72±.04^{ab}	Glycerol	0.44±.05^{b}	0.54±.15^{b}	0.2%	0.52±.08^{b}	5%	0.13±.03
		KNO_3	0.3± .15^{b}	N3(20)	0.88±.05^{a}	P3(1.0)	0.66±.05^{ab}	Maltose	0.33±.01^{ab}	0.62±.22^{b}	0.3%	0.28±.04^{a}		
		$NaNO_3$	0.87±.05^{c}	N4(10)	0.95±.06^{a}	P4(0.5)	0.564±.15^{a}	Acetic Acid	0.51±.21^{b}	0.14±.03^{a}	0.4%	0.36±.06^{a}	Normal	0.04±.08
5	**AIN63**	Urea	0.74±.10^{c}	N1(40)	0.77±.04^{ab}	P1(2.0)	0.61±.11^{c}	Glucose	0.15±.01^{a}	1.02±.25^{b}	0.1%	0.60±.13^{b}		
		NH_4Cl	0.13±.03^{a}	N2(30)	0.68±.04^{ab}	P2(1.5)	0.40±.14^{b}	Glycerol	0.58±.11^{c}	0.51±0.1^{a}	0.2%	0.23±.02^{a}	5%	0.95±.21
		KNO_3	0.36±.01^{b}	N3(20)	0.65±.08^{a}	P3(1.0)	0.29±.11^{ab}	Maltose	0.29±.02^{b}	0.51±.14^{a}	0.3%	0.20±.08^{a}		
		$NaNO_3$	0.65±.08^{c}	N4(10)	1.03±.03^{a}	P4(0.5)	0.18±.04^{a}	Acetic Acid	0.33±.07^{b}	0.53± 0.1^{a}	0.4%	0.17±.03^{a}	Normal	0.86±.04
6	**AIN68**	Urea	1.02±.14^{c}	N1(40)	0.82±.05^{a}	P1(2.0)	0.70±.11^{c}	Glucose	0.13±.02^{a}	0.92±.04^{a}	0.1%	0.66±.06^{b}		
		NH_4Cl	0.24±.01^{a}	N2(30)	0.79±.07^{a}	P2(1.5)	0.48±.16^{b}	Glycerol	0.36±.03^{c}	0.83±.04^{ab}	0.2%	0.33±.15^{a}	5%	0.77±.12
		KNO_3	0.29±.09^{a}	N3(20)	0.84±.02^{a}	P3(1.0)	0.33±.12^{ab}	Maltose	0.24±.02^{b}	0.89±.15^{a}	0.3%	0.34±.14^{a}		
		$NaNO_3$	0.84±.02^{b}	N4(10)	0.84±.08^{a}	P4(0.5)	0.23±.04^{a}	Acetic Acid	0.26±.04^{b}	0.7± 0.14^{a}	0.4%	0.32±.08^{a}	Normal	0.68±.04
7	**AIN79**	Urea	1.17±.16^{c}	N1(40)	1.22±.04^{a}	P1(2.0)	1.04±.15^{ab}	Glucose	0.11±.02^{a}	1.12±.23^{b}	0.1%	0.67±.12^{b}		
		NH_4Cl	0.21±.03^{a}	N2(30)	1.22±.09^{a}	P2(1.5)	1.16±.18^{b}	Glycerol	0.40±.11^{c}	0.81± 0.1^{a}	0.2%	0.40±.07^{a}	5%	0.98±.05
		KNO_3	0.82±.09^{b}	N3(20)	0.94±.02^{a}	P3(1.0)	0.97±.20^{ab}	Maltose	0.28±.01^{b}	0.74±.11^{a}	0.3%	0.37±.05^{a}		
		$NaNO_3$	0.94±.02^{c}	N4(10)	1.12±.04^{a}	P4(0.5)	0.85±.18^{a}	Acetic Acid	0.22±.04^{b}	0.7± 0.18^{a}	0.4%	0.36±.08^{a}	Normal	0.88±.01
8	**AIN85**	Urea	1.41±.25^{c}	N1(40)	1.70±.05^{b}	P1(2.0)	1.19±.30^{a}	Glucose	0.21±.07^{a}	0.8± 0.23^{a}	0.1%	0.76±.31^{a}		
		NH_4Cl	0.38±.05^{a}	N2(30)	1.50±.09^{a}	P2(1.5)	1.201±.37^{a}	Glycerol	0.41±.12^{b}	1.02±.26^{a}	0.2%	0.57±.13^{a}	5%	1.21±.15
		KNO_3	1.05±.10^{b}	N3(20)	1.49±.04^{a}	P3(1.0)	1.065±.26^{a}	Maltose	0.31±.03^{a}	0.89± 0.1^{a}	0.3%	0.55±.28^{a}		
		$NaNO_3$	1.49±.04^{c}	N4(10)	1.34±.04^{a}	P4(0.5)	1.011±.19^{a}	Acetic Acid	0.23±.04^{a}	1.06±.04^{a}	0.4%	0.62±.24^{a}	Normal	1.11±.01
9	**AIN93**	Urea	0.79±.11^{b}	N1(40)	1.19±.06^{a}	P1(2.0)	1.327±.07^{a}	Glucose	0.24±.09^{a}	0.52±.22^{b}	0.1%	1.04±.21^{a}		
		NH_4Cl	0.32±.03^{a}	N2(30)	1.21±.04^{a}	P2(1.5)	1.55±.05^{b}	Glycerol	0.41±0.1^{b}	0.67±.28^{b}	0.2%	1.03±.12^{a}	5%	1.01±.07
		KNO_3	1.27±.14^{c}	N3(20)	1.09±.05^{a}	P3(1.0)	1.41±.07^{a}	Maltose	0.31±.08^{ab}	1.0±.05^{a}	0.3%	1.25±.06^{a}		
		$NaNO_3$	1.09±.05^{c}	N4(10)	1.11±.04^{a}	P4(0.5)	1.331±.05^{a}	Acetic Acid	0.22±.06^{a}	1.01±.15^{a}	0.4%	1.11±.06^{b}	Normal	0.93±.04
10	**AIN95**	Urea	0.79±.07^{b}	N1(40)	1.13±.02^{b}	P1(2.0)	1.11±.05^{a}	Glucose	0.35±.03^{a}	1.11±.06^{b}	0.1%	1.12±.04^{c}		
		NH_4Cl	0.38±.04^{a}	N2(30)	0.93±.06^{a}	P2(1.5)	1.27±.12^{b}	Glycerol	0.44±.15^{a}	0.73±.15^{b}	0.2%	1± 0.01^{b}	5%	1.16±.03
		KNO_3	0.94±.01^{c}	N3(20)	0.98±.05^{ab}	P3(1.0)	1.07±.09^{a}	Maltose	0.30±.07^{a}	0.67±.06^{a}	0.3%	0.93±.01^{b}		
		$NaNO_3$	0.98±.05^{c}	N4(10)	1.01±.09^{ab}	P4(0.5)	1.15±.02^{ab}	Acetic Acid	0.34±.01^{a}	0.61±.05^{a}	0.4%	0.81±.06^{a}	Normal	1.06±.08

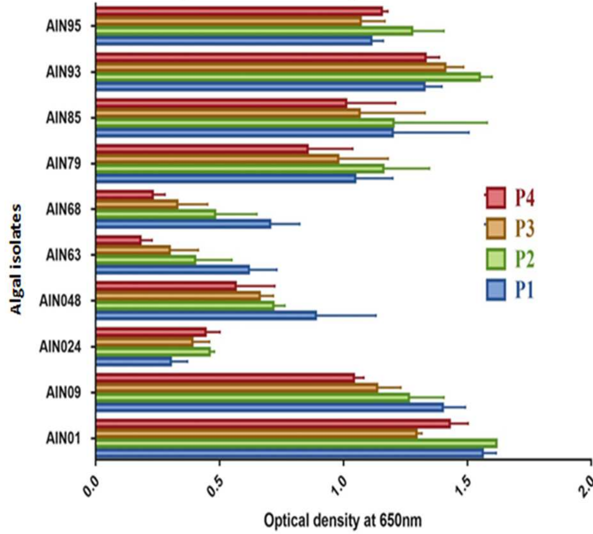
[a,b,c,d] Different superscript shows statistically significant difference ($p<0.05$) in different rows of same column.



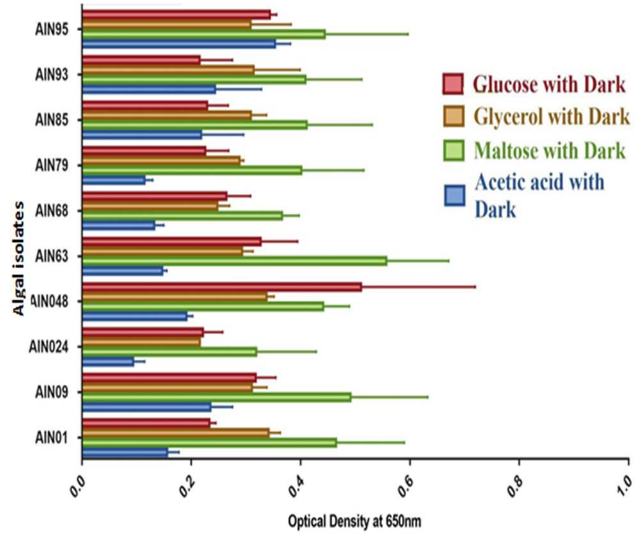
A. Effect of nitrogen sources on growth



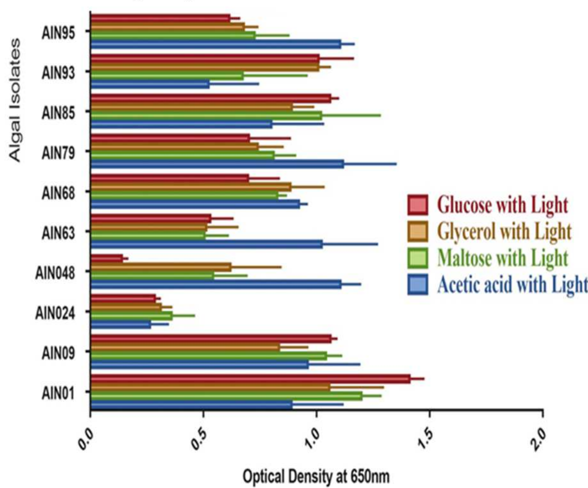
B. Effect of nitrogen concentrations on growth



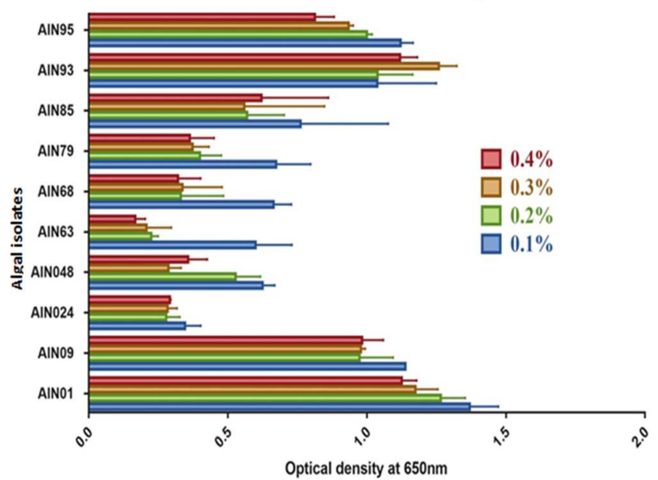
C. Effect of phosphorus concentrations



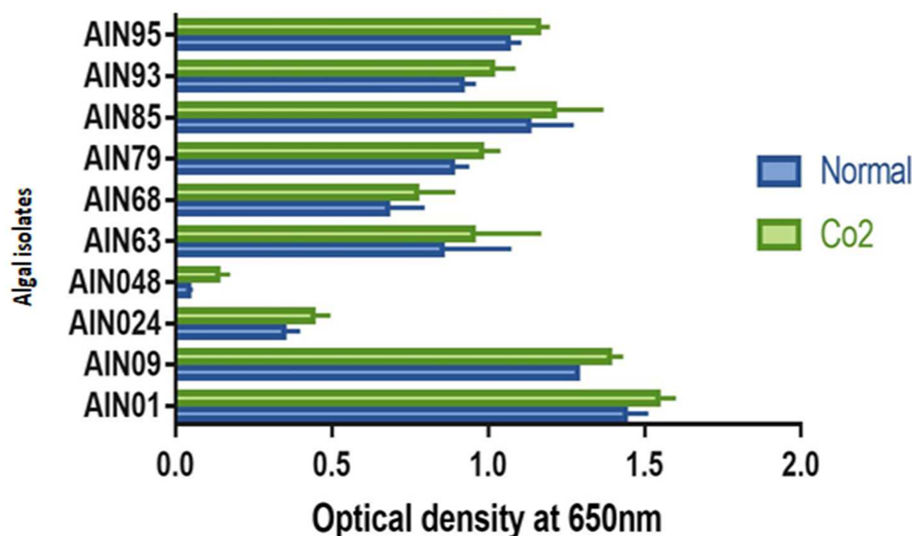
D. Effect of sources of carbon (absence of light)



E. Effect of carbon sources (presence of light)



F. Effect of different concentration of $NaHCO_3$



G. Effect of concentration of CO_2

Fig. 2. Effect of chemical factors on selected algal isolates at day 10 as determined by optical density (OD=650)

DISCUSSION

The current research focuses on assessing indigenous microalgae's bioenergy production potential. Physical and chemical factors play a major role in the growth of microalgae (Smith, 1986). Temperature is an important physical parameter that influences the growth and metabolism of microalgae (Richmond, 1986). Out of 10 isolates, three isolates, AIN63 (*Scenedesmus*), AIN93 (*Graesiella*), and AIN95 (*Chroococcus*), showed optimal growth at 25ºC. In our study the following isolates (AIN68, AIN 85), and (AIN01, AIN09, AIN79) showed optimum growth at 30ºC and 35ºC respectively. Zhu (2015) reported that for most microalga species, temperature should be kept between 20-30ºC to achieve ideal growth. In literature, many species of *Scenedesmus* and *Chlorella* can grow optimally at 30 and 35ºC. The pH change also affects the metabolic processes of microalgae (Wong and Franz, 2013). In our study, maximum algal isolates showed optimal growth only at pH 7. Olaizola (2003) reported that microalgae grow and tolerate pH 6 well. However, some researchers reported that microalgae grow well at pH 7 and 7.5 (Moheimani, 2013). Ren (2013) also studied that *Scenedesmus* grows well at pH 7. Alkaline and neutral pH increase the flexibility of the cell wall preventing the rupture of the cell walls of cells. So, there is an increase in the time to complete the cell cycle which leads to more biomass.

Maximum isolates (n=9) showed increased in biomass as the light intensities increased. Algal isolates (AIN85, AIN93) showed optimum growth at a light intensity of 1000 lux. As the light rises above the compensation point, it increases its growth till the light saturation point. As light intensity increases from the light saturation point, it does not affect biomass production (Zhu 2015). Most algal isolates showed satisfactory results at higher light intensities of 1500 and 2000lux. Takeshita *et al.* (2014) reported that *Chlorella* sp. increases its biomass with an increase in light intensity of 600 µmol photons $m^{-2}s^{-1}$. Studies also revealed that S*cenedesmus* and *Botryrococcus* increase their biomass as there is an increase in light intensity up to 6000 lux. Microalgae *Neochloris* can produce higher biomass to 14800 lux (Sun *et al.,* 2014).

The property of organisms to thrive best in a saline environment is the key to survival. As 03 isolates showed optimum growth at 0.5%, 04 isolates performed well at 1%, and 02 isolates at 2%. These results are in agreement with other researchers. Ibáñez *et al.* (2005) studied the impact of salinity on biomass production of *Dunaliella salina* and found an inverse relationship between salinity and growth. Andruleviciute *et al.* (2014) also studied the effect of salinities (30, 35, 40 g/L) on the growth of *Chlorella* and found that *Chlorella* thrives in all salinty levels, but biomass production is low as the sodium chloride concentraion increases. The decrease in microalgae growth at high sodium chloride levels is due to osmotic stress upon cells which tends to change their shape and metabolic processes and also affects the photosynthesis rate. These factors ultimately tend to decrease the growth rate of microalgae and hence reduce the biomass (Hasegawa *et al.,* 2000).

Nutrients play a vital role in the cultivation of microalgae. The three main nutrients (N, P and C) must be supplied in a medium quantity to produce more microalgae biomass (Pancha *et al.,* 2014). In order to optimize nitrogen sources, microalgal isolates were grown in four sources of nitrogen ($NaNO_3$, KNO_3, NH_4Cl, and Urea). In our study, a total of 03 isolates (AIN48, AIN85 and AIN95) respond well to $NaNO_3$.

While the 04 isolates showed the best growth at KNO_3, three isolates performed well in a medium supplemented with urea. In literature, different nitrogen sources (ammonium nitrate, urea, sodium nitrate, potassium nitrate, ammonium sulfate etc.) are tested for biomass production of microalgae (Mata *et al.,* 2010; Arumugam *et al.,* 2013). Harwati (2013) reported that micro-algal cultures grown in sodium nitrate produce more biomass than potassium nitrate and ammonium nitrate. Amin (2013) reported increased growth of Chlorella when supplemented with urea as compared to $NaNO_3$. Coban (2021) reported that KNO3 was the preferred N source for growth than $NaNO_3$ and urea. Some microalgae grow faster in NaNO3 while others, likes urea, as nitrogen sources. Requirement of Nitrogen source preference must be optimized for the proper culture of microalgal species.

Microalgae were grown in four different $NaNO_3$ concentration results indicating that 07 out of 10 showed the best biomass at 40 mM concentration. One strain showed optimal biomass production at 30mM. Morowvat and Ghasemi (2018) reported that *Chlorella* produces more biomass at higher concentrations (6g/L) of sodium nitrate than at lower concentrations. Similarly, Menegol *et al.* (2017) also reported that *Heterochlorella luteoviridis* produces more biomass at increasing nitrogen concentrations. Sharma *et al.* (2018) also studied the effect of the concentration of sodium nitrate on microalgae. They found that an increase in nitrogen level (1g/L) tends to increase biomass product, but lipids level decreases. Our finding suggests that more sodium nitrate concentration usually tends to increase in microalgae biomass. Still, some species do not respond well to increasing concentration of nitrogen source and they grow well at less sodium nitrate concentration due to its toxic effect. These finding agree with Costa *et al.* (2001), who stated that spirulina grows best at 0.01M and 0.02 M sodium nitrate, but as the concentration increases from 0.02 M, the biomass production of spirulina decreases.

In order to determine the effect of phosphorus concentration on the growth of microalgae, four different concentrations of phosphorus were utilized. Out of 10 isolates, 06 showed the highest growth at 2mM phosphorus. Only three isolates showed optimal growth at 1.5mM. The growth data indicates that the increase in phosphorus leads to increase in microalgae biomass, as Ruangsomboon *et al.* (2012) reported that *Chlorella* biomass and lipid contents increase with the increase in phosphorus concentration. Smith and Kalff (1981) reported that algal abundance in northeren lake varies with the concentration of phosphorus.

In our study, when our isolates were grown heterotrophically (without light and the presence of carbon sources), 10 microalgal strains showed optimal growth at 1% glycerol level rather than acetic acid, maltose, and glucose. Wu *et al.* (1994) reported that *Chlorella* produces higher biomass when grown heterotrophically using glucose, acetate, or other carbon compounds. However, when microalgae were grown mixotrophically in BG-11 medium containing glucose, glycerol, maltose and acetic acid, five strains optimally performed at 1% glucose, 01 and 4 strains optimally performed at 1% glycerol and acetic acid, respectively. These results agree with other research data, as Harwati (2013) reported that C*hlorella* grown in mixotrophic conditions produces more biomass. He also found that *Chlorella* when supplied with acetic acid as a carbon source has more biomass than glucose.

Like plants, microalgae can fix CO_2 from the environment into useful compounds. However, in some situations, obtaining a good source of CO_2 is difficult. To enhance biomass production by providing a stable supply of CO_2, sodium bicarbonate at different concentrations was used. In our study, a maximum number of isolates (n=09) showed optimal growth at 0.1%, and only 01 isolate showed optimal growth at 0.2% sodium bicarbonate only. Lee *et al.* (2003) reported that C*hlorella* grows optimally at 15 ppm. The availability of carbon dioxide greatly affects the biomass production of microalgae. All isolates showed more biomass production at 4% CO_2 than normal CO_2 in the atmosphere. These results agree with other research work, as Moheimani (2013) obtained the highest biomass of *Chlorella* and *Tetraselims* by utilizing CO_2 as a carbon source. The present study and earlier studies revealed that optimal physicochemical parameters are significantly appropriate for maximum microalgal biomass production at a large scale. In our research, microalgae AIN01 (*Chlorella*) performs significantly well in terms of growth as compared to other isolates. Moreover, optimal growth of microalgae can be achieved at 30°C and 35°C temperatures, 6 and 7 pH, light intensity of 1000 lux, 0.5% NaCl concentration, sodium nitrate as nitrogen source, 40 mM concentration of $NaNO_3$, 2mM phosphorus concentration, heterotrophically (absence of light and presence of carbon sources) at 1% glycerol level, mixotrophically at 1% glucose concentration, 0.1% sodium bicarbonate concentration and in 4% CO_2 then atmospheric CO_2.

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Author contributions: MI Najeeb performed research work and AA Anjum was involved in conceptualization of research study. MD Ahmad and A Maqbool guided about conducting experiments and validation of results. MI Najeeb, T Ali and R Manzoor involved in writing-original draft, its improvement and submission.

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